# ENZYME METHOD FOR DETECTING LYSOPHOSPHOLIPIDS AND PHOSPHOLIPIDS AND FOR DETECTING AND CORRELATING CONDITIONS ASSOCIATED WITH ALTERED LEVELS OF LYSOPHOSPHOLIPIDS

## RELATED APPLICATION INFORMATION

This application is a continuation-in-part of U.S. Patent Application Serial No. 09/176,813 filed October 12, 1998. The priority of the prior application is expressly claimed, and the disclosure of this prior application is hereby incorporated by reference in its entirety.

## FIELD OF THE INVENTION

The present invention relates to enzyme methods for detecting lysophospholipids, such as lysophosphatidic acid, (LysoPA) and lysophosphatidyl choline (LysoPC), in biological fluids, and for correlating and detecting conditions associated with altered levels of lysophospholipids.

## BACKGROUND OF THE INVENTION

Phosphatidyl choline (PC), also named lecithin, is one of the major sources of polyunsaturated fatty acids such as arachidonic and linoleic acids. The former is a precursor of eicosanoids which have numerous biological activities. Hydrolysis of PC yields lysophosphatidyl choline (LysoPC) and constituent fatty acids, which have been implicated in signal transduction (Asaoka et al., Proc. Natl. Acad. Sci. USA 90:4917-4921 (1993); Yoshida et al., Proc. Natl. Acad. Sci. USA 89:6443-6446 (1992)). An increasing body of evidence indicates that LysoPC, which is present in high concentrations in oxidized low density lipoproteins may play a significant role in atherogenesis and other inflammatory disorders (Steinberg et al., New..Eng. J. Med. 320:915-924 (1989)). LysoPC has been reported to increase the transcription

of genes encoding platelet derived growth factor A and B chains, and heparin-binding epidermal growth factor-like protein (HB-EGF) in cultured endothelial cells (Kume and Gimbrone, J. Clin. Invest. 93:907-911 (1994)), and to increase mRNA encoding HB-EGF in human monocytes (Nakano et al., Proc. Natl. Acad. Sci. USA 91:1069-1073 (1994)). These gene products are mitogens for smooth muscle cells and fibroblasts (Higashiyama et al., Science 251:936-939 (1991); Ross, Nature (Lond.) 362:801-809 (1993)). LysoPC has also been shown to activate protein kinase C in vitro (Sasaki et al., FEBS Letter 320:47-51 (1993)), to potentiate the activation of human T lymphocytes (Asaoka et al., Proc. Natl. Acad. Sci. USA 89:6447-6451 (1992)) and to potentiate the differentiation of HL-60 cells to macrophages induced by either membrane-permeable diacylglycerols or phorbol esters (Asaoka et al., Proc. Natl. Acad. Sci. USA 90:4917-4921 (1993)).

LysoPC may also provide a source of bioactive lysophosphatidic acid (1-acyl-sn-glycero-3-phosphate, LysoPA) (Moolenaar et al., Rev. Physiol. Biochem. Pharmacol. 119:47-65 (1992)) through hydrolysis by lysophospholipase D (Tokumara et al., Biochim. Biophvs. Acta 875:31-38 (1986)). LysoPA is a naturally occurring phospholipid with a wide range of growth factor-like biological activities. It is well established that LysoPA can act as a precursor of phospholipid biosynthesis in both eukaryotic and prokaryotic cells (Van den Bosch, Ann. Rev. Biochem. 43:243-277 (1974); Racenis et al., J. Bacteriol. 174:5702-5710 (1992)). The ability of LysoPA to act as an intercellular lipid mediator has been noted (Vogt, Arch. Pathol. Pharmakol. 240:124-139 (1960); Xu et al., J. Cell. Physiol. 163:441-450 (1995); Xu et al., Biochemistry 309:933-940 (1995); Tigyi et al., Cell Biol. 91:1908-1912 (1994); Panetti et al., J. Lab. Clin. Med. 129(2):208-

216 (1997)). LysoPA is rapidly generated by activated platelets and can stimulate platelet aggregation and wound repair.

Ovarian cancer activating factor (OCAF), has been isolated from ovarian cancer ascites fluid (Mills et al., Cancer Res. 48:1066 (1988); Mills et al. J. Clin. Invest. 86:851 (1990) and U.S. Patent Nos. 5,326,690 and 5,277,917) and has been identified to consist of multiple forms of LysoPA (Xu et al., Clin. Cancer Res. 1:1223-1232 (1995)). LysoPA has been identified as a potent tumor growth factor in the ascites fluid of ovarian cancer patients *(Id.)*

Other lysophospholipids associated with various conditions include lysophosphatidyl 20 serine (LysoPS), lysophosphatidyl ethanolamine (LysoPE), lysophosphatidyl glycerol (LysoPG and lysophosphatidyl inositol (LysoPI). Activated platelets secrete two kinds of phospholipase: sPLA2 and PS-PLA1. sPLA2 is reported to be elevated in inflammatory reactions and inhibition of this enzyme reduced inflammation (Schrier et al., Arthritis Rheum. 39(8):1292-1299 (1996); Tramposch et al., Pharmacol. and Experimental Therapeutics 271 (2):852-859 (1994)). PS-PLA1 hydrolyzes phosphatidylserine or lysophosphatidyl seine (LysoPS) specifically to produce LysoPS or Glycerol-3-P serine. LysoPS strongly enhances degranulation of rat mast cells induced by concanavalin A and potentiates histamine release (Tamori-Natori et al., J. Biochem (Tokyo) 100(3):581-590 (1986)), and can stimulate sPLA2-elicited histamine release from rat serosal mast cells (Hara et al., Biol. Pharm. Bull. 19(3):474-476 (1996)). LysoPS is an inflammatory lipid mediator (Lloret et al., J. Cell Physiol. 165(1):89-95 (1995)) and sPLA2 has been implicated in inflammation processes (Lloret et al., Toxicon 32(11):1327-1336 (1994)). LysoPI has been shown to stimulate yeast adenylyl cyclase activity with implications for

modulating the activity of downstream effector molecules and their interaction with RAS proteins (Resnick and Thomaska,, J. Biol. Chem. 269(51):32336-32341 (1994)).

Methods for separating and semi-quantitatively measuring phospholipids such as LysoPA using techniques such as thin-layer chromatography (TLC) followed by gas chromatography (GC) and/or mass spectrometry (MS) are known. For example, lipids may be extracted from the test sample of bodily fluid using extraction procedures such as those described by Bligh and Dyer, Can. J. Biochem. Physiol. 37:911-917 (1959). Thin-layer chromatography may be used to separate various phospholipids, for example as described by Thomas and Holub, Biochim. Biophys. Acta. 1081:92-98 (1991). Phospholipids and lysophospholipids are then visualized on plates, for example using ultraviolet light as described by Gaudette et al., J. Biol. Chem. 268:13773-13776 (1993). Alternatively, lysophospholipid concentrations can be identified by NMR or HPLC following isolation from phospholipids or as part of the phospholipid (Creer and Gross, Lipids 20(12):922-928 (1985) and Bowes et al., J. Biol. Chem. 268(19)13885-13892 (1993)). LysoPA levels have also been determined in ascites from ovarian cancer patients using an assay that relies on LysoPA-specific effects on eukaryotic cells in culture (Mills et al., Cancer Res. 48:1066-1071 (1988)). However, these prior procedures are time-consuming, expensive and variable and typically only semi-quantitative.

Development of a rapid and sensitive assay for lysophospholipid species would facilitate use of these lysophospholipids as markers for cellular activities such as platelet activation and for conditions associated with altered levels of lysophospholipid species. Moreover, such assays would provide a method for determining correlations between altered levels of a

lysophospholipid and conditions associated with such altered levels.

## SUMMARY OF THE INVENTION

The present invention encompasses enzymatic methods for determining concentrations of lysophospholipids, such as LysoPA, in samples of biological fluids such as serum or plasma. The methods involves a two-step enzymatic digestion of at least one type of lysophospholipid to produce a substrate for a subsequent enzymatic reaction which produces a detectable end product that then permits detection of the concentration of the lysophospholipid.

The methods are carried out by detecting the concentration of a lysophospholipid such as LysoPA in a sample of bodily fluid taken from a subject. The lysophospholipid in the sample is preferably first enriched through extraction of lipids. For example, polar lipids are redissolved in aqueous solution and the concentration of lysophospholipid is determined using a two-step enzymatic reaction. The lysophospholipid is digested using an enzyme to generate a product that is then subject to a second enzymatic reaction. In a specific embodiment, the second reaction is an enzymatic cycling reaction that amplifies the signal. This method permits measurement of a lysophospholipid present in small amounts in the test sample.

In one embodiment, an enzyme such as lysophospholipase or phospholipase B is used to liberate G3P from LysoPA. The level of G3P is determined using an enzymatic cycling reaction that employs G3P oxidase and glycerol-3-phosphate dehydrogenase in the presence of NADH. The amount of LysoPA detected is quantitated spectrophotometrically by measuring the oxidation of NADH. Alternatively, the amount of LysoPA is determined colorimetrically by

detection of hydrogen peroxide generated by the cycling reaction.

In addition to LysoPA, other lysophospholipids such as LysoPC, lysophosphatidyl serine (LysoPS), lysophosphatidyl inositol (LysoPI), lysophosphatidyl ethanolamine (LysoPE) and lysophosphatidyl glycerol (LysoPG), can be detected using the methods of the invention. For these lysophospholipids, alternative enzymes for use in the methods include, but are not limited to, phospholipase $A_1$, phospholipase $A_2$, phospholipase C, phospholipase D, lecithinase B and lysolecithinase, glycerophosphocholine phosphodiesterase and glycerol kinase.

The enzymatic methods of the invention can be used to detect altered levels of lysophospholipid in a subject compared to normal levels of the lysophospholipid in normal to detect conditions associated with such altered levels of lysophospholipid. Diagnosis of a condition using the methods of the invention may also be performed by determining the rate of change over time of the concentration of a lysophospholipid in samples taken from the subject.

Another embodiment of the invention is use of the assay in a method to determine whether a correlation exists between the level of a lysophospholipid and the presence of a condition. In this embodiment, the concentration of a lysophospholipid is determined in samples from subjects known to have a specific disease condition, such as an inflammatory condition, and compared to concentration of that lysophospholipid in subjects free of such condition. Altered levels of lysophospholipid in the samples from the subjects having a condition as compared to samples from normal subjects suggest a correlation between the levels of the lysophospholipid and the presence of the condition.

Yet another embodiment of the methods of the invention is a diagnostic kit containing

enzyme and other reagents for conducting the enzymatic assays of the invention to measure concentrations of lysophospholipids in samples of bodily fluids taken from subjects.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides enzymatic methods for detecting and quantifying altered concentrations of lysophospholipids, including, but not limited to, lysophosphatidic acid (LysoPA), lysophosphatidyl choline (LysoPC), lysophophatidyl serine (LysoPS), lysophosphatidyl inositol (LysoPI), lysophosphatidyl ethanolamine (LysoPE) and lysophosphatidyl glycerol (LysoPG) in a sample of bodily fluid from a subject.

The subject is an eukaryotic organism, preferably a vertebrae, including, but not limited to, a mammal, a bird, a fish, an amphibium, or a reptile. Preferably, the subject is a mammal, most preferably a human. The bodily fluid includes, but is not limited to, plasma, serum, urine, saliva, ascites, cerebral spinal fluid or pleural fluid.

The conditions correlated with altered concentrations of these lysophospholipids include, but are not limited to, inflammatory conditions, *i.e.* conditions associated with platelet activation. Altered phospholipid metabolism has been reported in a number of diseases (for review see Gregor Cevc (Ed.), Phospholipids Handbook, Ch. 28: Gupta, Phospholipids in Disease, pp. 895-908 (1993)) and can lead to altered lysophospholipid and phospholipid levels in biological fluids. These diseases include, but are not limited to, sickle cell anemia, diabetes, muscular dystrophy, ischemia, liver disease, lung disease, heart disease, malaria, Alzheimer's, Parkinson's and various cancers. In these conditions, defective cellular functions may directly or indirectly lead

to changes in steady state levels of phospholipids. Other diseases include bleeding disorders including those associated with abnormal platelet function resulting in coagulopathy.

Thus, the methods of the present invention are directed to the detection of conditions that are known to correlate, or the identification of conditions to correlate, with altered concentrations of lysophospholipids in the bodily fluids from a subject relative to concentrations found in bodily fluids from a subject lacking a condition associated with altered concentrations of lysophospholipids *(i.e.* "normal subjects").

USES OF THE INVENTION

The methods of the invention provides a rapid and accurate assay with increased sensitivity for detecting small amounts of lysophospholipids present in samples of bodily fluids from subjects. The enzymatic assay can be used to detect conditions associated with altered levels of lysophospholipids in a sample from a subject as compared to normal samples. In addition, the assay permits determination of correlations between various disease conditions and alterations in the levels of lysophospholipids. The methods of the invention and test kits thus provide a practical means to detect conditions associated with altered levels of certain lysophospholipids.

ENZYMATIC METHODS FOR DETECTING AND OUANTIFY1NG LYSOPHOSPHOLIPIDS

The methods of the invention are carried out as follows. A biological sample such as whole blood is collected from a subject. Lipids are extracted from plasma or serum from the

sample, for example, by organic extraction using chloroform:methanol and centrifugation and enriching for a selected species of lysophospholipid, *e.g.* LysoPA, or for total lysophospholipids. The need for enrichment depends in part on the specificity of the enzyme used to digest the lysophospholipid to be determined. An enzyme which hydrolyzes the lysophospholipid is incubated with the extracted lipid sample producing a smaller metabolite. Next another enzymatic digestion is performed to produce a detectable product. In one embodiment an enzyme cycling reaction which consists of two enzymatic reactions that accumulates detectable products is performed. In the Examples herein to detect LysoPA levels, Phospholipase B (PLB) or lysophospholipase (LYPL, EC 3.1.1.5, Asahi Chemical Industry Co., Ltd., Tokyo, Japan) is used to produce glycerol-3-phosphate (G-3-P). An enzyme cycling reaction is then performed using glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate oxidase and NADH to accumlate $H_20_2$ and NAD (U.S. Patent No. 5,122,454, Ueda et al.)

The level of LysoPA is detected by monitoring the oxidation of NADH spectrophotometrically at 340 nm *(i.e.* disappearance of $0D_{340}$) and the accumulation of $H_20_2$ colorimetrically using peroxidase. Numerical values are obtained from a standard curve consisting of known C18:1 LysoPA. Typical standard curves include known amounts of LysoPA from 0 to 3 μM. Assays are preferably performed in duplicate with both positive and negative controls. The difference between $OD_{340}$ before and after the enzyme cycling reaction is directly proportional to the amount of LysoPA present. Background signals in plasma without phospholipase B are substracted from all samples. LysoPA standard curve values are plotted and fitted to a linear or second-order polynominal curve fit. The levels of LysoPA in each sample are

determined by comparing each signal measured to the standard curve.

Alternatively, the lysophospholipid can be detected using additional and/or different enzyme combinations. For example, phospholipase C (BC 3.1.4.3, Sigma Chemical Co., St. Louis, MO) is used to cleave inorganic phosphate (Pi) from LysoPA. Levels of LysoPA are then determined by measuring the amount of liberated Pi using established procedures, *e.g.* using a commercially available kit (Procedure 670, Sigma Chemical Co., St. Louis, MO). For increased sensitivity, Pi is determined using purine nucleoside phosphorylase (PNP), xanthine oxidase (XOD) and urate oxidase (UOD) as previously described (Kawasaki et al., Analytical Biochem. 182:366-370 (1989)). The latter method generates 3 $H_20_2$ molecules for every Pi. The accumulation of $H_20_2$ is detected colorimetrically using peroxidase.

In another embodiment, the lysophospholipid, such as LysoPA, is incubated with phospholipase B or lysophospholipase to produce G-3-P. G-3-P is converted to dihydroxyacetone phosphate and hydrogen peroxide using G-3-P oxidase in the presence of oxygen and water. In the presence of NADH, G-3-P dehydrogenase converts dihydroxyacetone phosphate back to G-3-P and oxidizes NADH to NAD. The disappearance of NADH is monitored spectrophotometrically at $OD_{340}$. Alternatively, the production of hydrogen peroxide may be measured, for example colorimetrically by fluorometry or chemiluminescence. For a colorimetric assay any of a number of chromogenic substrates may be used including 4-aminoantipyrine (AAP), pyrogallol, 2-(2'-Azinobis (3-ethylbenzthiazoline-sulfonic acid) (ABTS) and 3,3',5,5'-tetramethylbenzidine) (TMB).

In yet another embodiment, LysoPC may be determined by liberating

glycerophosphorylcholine (GPC) and fatty acid from LysoPC using phospholipase B or lysophospholipase. The level of LysoPC is determined by liberating choline and glycero-3-phosphate (G-3-P) from GPC using GPC phosphodiesterase (GPC-PDE) followed by a colorimetric enzymatic determination of choline using choline oxidase, 4-aminoantipyrine (AAP), 3,5 Dichloro-2-hydroxybenzenesulfonic acid sodium salt (HDCBS) and peroxidase. Choline is detected by oxidizing to $H_2O_2$ and betaine and then using peroxidase to form quinoneimine dye. Alternatively, G-3-P is measured using G-3-P dehydrogenase and oxidase in the cycling reaction of the assay of the invention.

In addition to LysoPA and LysoPC, other lysophospholipids such as lysophosphatidyl serine (LysoPS), lysophosphatidyl inositol (LysoPI), lysophosphatidyl ethanolamine (LysoPE) and lysophosphatidyl glycerol (LysoPG), can be detected using the two step enzymatic assay methods of the invention.

Enzymes for use in the first step of the method to digest lysophospholipids include, but are not limited to, lysophospholipase, phospholipase B, phospholipase $A_1$, phospholipase $A_2$, phospholipase C, and phospholipase D.

Enzymes for use in detecting the product of enzymatic digestion of lysophospholipids in step one include glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate oxidase, glycerophosphorylcholine phosphodiesterase (GPC-PDE), choline oxidase, serine dehydrogenase, serine deaminase, aldehyde dehydrogenase, ethanolamine deaminase, glycerokinase and glycerol dehydrogenase.

For example, to determine LysoPS, the LysoPS is digested by phospholipase D into

serine and LysoPA. The amount of serine produced is determined by detecting NADH formation (absorbance at $A_{340}$) via serine dehydrogenase. Alternatively, the serine is deaminated using deaminase to form ammonia ($NH_3$) and $HOCH_2$-CO-COOH. Alternatively, LysoPS can be digested by lysophospholipase to form Glycerol-3-P serine which is then digested using glycerol-3-P choline phosphodiesterase (GPC-PDE) to form Glycerol-3-P and serine. The LysoPS is determined by detecting $NH_3$ production or NADPH production via serine dehydrogenase or by using a Lyso-PS specific lysophospholipase enzyme.

LysoPE can be determined using the enzyme assay of the invention by hydrolyzing LysoPE into LysoPA and ethanolamine by phospholipase D. The ethanolamine is then deaminated by deaminase and dehydrogenated to produce NADH to produce $HOCH_2$-CHO and $NH_3$ The $HOCH_2$-CHO is then digested with aldehyde dehyrogenase to form NADH which is then detected by spectrometry (*e.g.* at $A_{340}$). Alternatively a LysoPE-specific lysophospholipase enzyme can be used to hydrolyze LysoPE to Glycerol-3-P ethanolamine which in turn is hydrolyzed to Glycerol-3-P by glycerophosphorylcholine phosphodiesterase (GPC-PDE). Glycerol-3-P is then measured using the cycling reaction of the invention.

In the methods of the invention, an alternative to the liquid organic extraction for enrichment includes the use of solid phase extraction using, *e.g.* a Bond-Elut® column (Varian, Harbor City, CA) consisting of silica or fluorosil can be used to enrich for the lysophospholipid and to remove proteins and other lipids.

In order to optimize recovery of the desired lysophospholipid, inhibitors of endogenous enzymes that may be present in the sample may be used to prevent an increase in background

levels of lysophospholpid or degradation of the lysophospholipid levels in the sample. Such inhibitors include specific $PLA_2$ inhibitors such as Aristolic Acid (9-methoxy-6-nitrophenanthro-(3 ,4-d)-dioxole-5-carboxylic acid, Biomol Research Laboratories, Plymouth Meeting, PA); ONO-R-082 (2-(p-Amylcinnamoyl)amino-4-chlorobenzoic acid, Biomol); OBAA (3-(4-Octadecyl)-benzoylacrylic acid, Biomol), 4-Bromophenacyl Bromide (Sigma); Quincrine (6-Chloro-9-(4-diethylamino)-1-methylbutyl)amino-2-methoxycridine, Mepacrine, Sigma); Manoalide (Biomol) and HELSS (Haloenol lactone suicide substrate, Biomol); phosphodiesterase inhibitors such as IBMX (3-Isobutyl- 1 -methylxanthine, CalBiochem, La Jolla, CA); Ro-20- 1724 (CalBiochem); Zaprinast (CalBiochem) and Pentoxifylline (CalBiochem); general protease inhibitors such as E-64 (trans-Epoxysuccinyl-L-leucylamido-(4-guanidino) butane, Sigma); leupeptin (Sigma); pepstatin A (Sigma); TPCK (N-tosyl-L-phenylalanine chloromethyl ketone, Sigma); PMSF (Phenylmethanesulfonyl fluoride, Sigma); benzamidine (Sigma) and 1,10-phenanthroline (Sigma); organic solvents including chloroform and methanol; detergents such as SDS; proteases that would degrade phospholipases such as trypsin (Sigma) and thermostable protease (Boehringer Mannheim Biochemicals, Indianapolis, IN); and metal chelators such as EDTA (Ethylenediaminetetracetic acid, Sigma) and EGTA (Ethylene glycol-bis-(beta-aminoethyl ether), Sigma).

The assay may be performed in a microtiter plate format to permit small volumes of samples and reagents to be employed and for monitoring, *e.g.* using an ELISA reader. These formats facilitate automating the performance of the assay. Reduced processing times for the assays using such formats may reduce variability between results.

## CORRELATION OF LYSOPHOSPHOLIPID LEVELS WITH DISEASE

Initially, physiological ("normal") concentrations of lysophospholipids and/or specific lysophospholipid species are determined in subjects not having a disease condition. Subsequently, the concentration of the lysophospholipids are measured in a sample of bodily fluid from a test subject to be screened for the disease and compared to the concentrations established for normal subjects. Concentrations of lysophospholipid that are significantly increased or decreased relative to normal controls, for example one or more standard deviations above normal, may indicate the presence of a condition associated with altered levels of the lysophospholipid.

In addition, the response of a condition to treatment may be monitored by determining concentrations of lysophospholipid in samples taken from a subject over time. The concentration of a lysophospholipid is measured and compared to the concentration taken at the earlier time from that patient. If there is an increase in the concentration of lysophospholipid over time, it may indicate an increase in the severity of the condition in the subject. Conversely, if there is a decrease in the concentration of lysophospholipid, it may indicate an improvement in the condition of the subject.

## DIAGNOSTIC KITS

The methods described herein for measuring concentrations of lysophospholipids in samples of bodily fluids from a subject may also be performed, for example, by using pre-packaged diagnostic kits. Such kits include enzyme reagents for digesting one or more

lysophospholipid, for example phospholipase B. The reagents include those to perform the enzyme cycling reaction such as glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate oxidase and β-nicotinamide adenine dinucleotide (NADH) and ancillary agents such as buffering agents, and agents such as EDTA to inhibit subsequent production or hydrolysis of lysophospholipids during transport or storage of the samples. The kits may also include an apparatus or container for conducting the methods of the invention and/or transferring samples to a diagnostic laboratory for processing, as well as suitable instructions for carrying out the methods of the invention.

The following examples are presented to demonstrate the methods of the present invention and to assist one of ordinary skill in using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure or the protection granted by Letters Patent granted hereon.

## EXAMPLES

### EXAMPLE I

### DETECTION AND QUANTITATION OF LYSOPA LEVELS IN HUMAN PLASMA

#### Reagents

Phospholipase B (PLB), glycerol-3-phosphate oxidase, glycerol-3-phosphate dehydrogenase, human plasma, human serum, 4-aminoantipyrine (AAP) and calcium chloride were purchased from Sigma Chemical Co., St. Louis, MO. Lysopholipase (LYPL) was purchased from Asahi Chemical Industry, Tokyo, Japan. Peroxidase and NADH were purchased

from Boerhinger Mannheim, Indianapolis, IL. All lipid standards, fatty acids and methyl esters were purchased from Avanti Polar Lipids, Alabaster, AL or Sigma Chemical Co. 3,5 Dichloro-2-hydroxybenzenesulfonic acid sodium salt (HDCBS) was purchased from Biosynth AG, Naperville, IL.

Sample Collection and Processing

Blood was collected in BD vacutainer tubes #6415 or #7714 utilizing a 3.2% buffered citrate (acid citrate) and maintained capped on ice until processing. Within 1 hour of draw, blood was centrifuged at 3000 x g (in a cold centrifuge if possible) for 15 minutes. Plasma was removed and transferred to a plastic tube and frozen at -20$^0$C to -80$^0$C. Alternatively, blood was drawn into EDTA-containing vacutainer tubes and centrifuged at 580 x g for 5 minutes. The supernatant was transferred to a siliconized tube and centrifuged again at 8000 x g for 5 minutes. The supernatant was collected into another siliconized tube and frozen at -70 $^0$C.

Sample Preparation and Thin Layer Chromatography

Approximately 0.5 ml of plasma was added to 3.75 ml of chloroform:methanol (1:2), vortexed and centrifuged at 3000 rpm for 10 minutes. The supernate was decanted into a new tube to which was added 1.25 ml chloroform and 1.75 ml water. This mixture was vortexed and centrifuged again to yield a biphasic solution. The lower layer was saved and the upper layer was collected into another tube. To this upper layer, 2.5 ml chloroform and 63 $\mu$l concentrated hydrochloric acid were added. The mixture was vortexed and then centrifuged again. The lower layer resulting from this acidified chloroform extraction was collected and pooled with the lower

layer that was saved. The pooled extract volume was reduced to less than 50 $\mu$l under a nitrogen stream and spotted onto the origin of a silica gel G TLC plate (Fisher Scientific, Santa Clara, CA). Chromatography was performed in a solvent system containing chloroform:methanol:ammonium hydroxide (65:35:5.5).

Lipids and standards were visualized by spraying the developed plate with Rhodamine 6G (Sigma Chemical) in water and the spot corresponding to LysoPA was scraped from the plate. Each sample was spiked with heptadecanoic acid as an internal standard. The fatty acids were hydrolyzed by adding 1 ml of 1N NaOH in methanol and incubating at $100^0$C for 15 minutes. After cooling, 1 ml of boron triflouride (14% in methanol, Alltech Associates, Deerfield, IL) was added and the sample incubated 30 minutes at room temperature to produce methyl esters. 2 ml hexane and 1 ml water were added and the mixture was vortexed thoroughly and centrifuged for 3-5 minutes at 3000 rpm to facilitate phase separation. The organic (top) layer was collected, dried under nitrogen, resuspended in 25 $\mu$l hexane and sealed in an autosampler vial.

Gas Chromatography

Fatty acid methyl esters (FAMES) were quantified using gas chromatography (GC) on a Hewlett Packard 5890 Series II GC fitted with an autosampler and flame ionization detector. 2 $\mu$l of sample in hexane were injected into a Supelco S PB-5 capillary column (Supelco, Bellefonte, PA). The GC program was set as follows: 170-$235^0$C at $10^0$C per minute and then held at $235^0$C for 13.5 minutes for a total run time of 20 minutes. Retention times for the methyl

esters were determined using known standards and compared to peaks in unknown samples. Quantitation of peaks was performed by comparison to a heptadeconic acid standard curve using calibration against the heptadecanoic acid internal standard.

Sample Preparation For The Enzymatic Assay

Approximately 0.5 ml of plasma were added to 3.75 ml of chloroform:methanol (1:2), vortexed and centrifuged at 3000 rpm for 10 minutes. The supernate was decanted into a new tube to which was added 1.25 ml chloroform and 1.75 ml water. This mixture was vortexed and centrifuged as above to yield a biphasic solution. The upper layer was collected into another tube and 2.5 ml chloroform and 63 $\mu$l concentrated hydrochloric acid were added, the mixture vortexed and centrifuged as before. The lower layer was collected and transferred into a clean tube. The sample was evaporated completely under nitrogen and the dried lipid extract was reconstituted in 250 $\mu$l of sample buffer containing 2.5% Triton X-100, 5 mM $CaCl_2$, and 100 mM Tris (pH 8.0). The sample was stored at -70$^0$C until it was assayed.

Alternatively, a modified extraction procedure was developed that only utilized 100 $\mu$l of sample and significantly reduced the levels of contaminating lipids such as phosphatidylcholine and lysophosphatidylcholine. In this extraction, 0.1 ml of plasma was added to 0.75 ml of chloroform:methanol (1:2), vortexed and centrifuged at 14,000 rpm for 5 minutes. The supernate was decanted into a new tube to which was added 0.25 ml of chloroform and 0.35 ml of water. This mixture was vortexed and centrifuged as above to yield a biphasic solution. The lower layer was discarded and to the remaining upper layer was added 0.5 ml chloroform. The sample was vortexed and centrifuged again at 14,000 rpm for 5 minutes. Once again the lower layer was

discarded. To the upper layer, 0.5 ml chloroform and 12.6 $\mu$l concentrated hydrochloric acid were added, the mixture vortexed and centrifuged as before. The acidified lower layer was collected and transferred to a clean tube. The sample was evaporated completely under nitrogen and reconstituted in 100 $\mu$l of sample buffer containing 2.5% Triton X-100, 5 mM $CaCl_2$, and 100 mM Tris (pH 8.0). The sample was stored at -70$^0$C until assayed.

Enzyme Assay

In the well of a 96 well microtiter plate, 5 – 100 $\mu$l of the extracted lipid sample was incubated with 0.25 units of phospholipase B or LYPL in 100 mM Tris (pH 8.0) at 37$^0$C for 30-60 minutes to produce G-3-P: 100 $\mu$/1 of cycling reaction enzyme mix containing 1.7 units of G-3-P dehydrogenase, 4 units of G-3-P oxidase, 0.25 mM NADH and 5 mM $CaCl_2$ in 50 mM Tris (pH 8.0) was added and the mixture incubated at 37$^0$C for an additional 60 minutes. The G-3-P oxidase converts G-3-P to dihydroxyacetone phosphate and $H_20_2$. The dihydroxyacetone phosphate is in turn converted back to G-3-P by G-3-P dehydrogenase. This reaction oxidizes NADH to NAD. Therefore, as cycling continues, both $H_20_2$ and NAD accumulate.

The level of LysoPA was determined by monitoring the oxidation of NADH *(i.e.* the reduction of absorbance at 340 nm after the cycling action compared to $A_{340}$ before cycling). In addition, the accumulation of $H_20_2$ was determined colorimetrically by adding 50 $\mu$l of a solution containing 0.5 units peroxidase, 0.5% HDCBS and 0.15% AAP in 100 mM Tris 8.0 to each well and recording the absorbance at 505 nm.

Numerical values for concentrations of LysoPA were obtained from a standard curve constructed from known LysoPA amounts. An internal standard of extracted plasma was

included within each assay (*i.e.* each plate) that was measured at different dilutions. In some cases, this internal standard was used to correct for variations between different experiments. Internal standards were also measured in the absence of PLB or LYPL enzyme. This "no-enzyme" sample provided a background value that was subtracted from each unknown when calculating the LysoPA levels using the NADH measurement. When the colorimetric method was used, the plate was blanked at 505 nm prior to color development.

Results

The results of the two-step enzymatic assay of the invention are shown in Table 1.

**TABLE I**

**ENZYME ASSAY TO DETECT LYSOPA**

| | Enzyme Assay | TLC/GC Assay |
|---|---|---|
| Sensitivity | 0.2 $\mu$M | 1 $\mu$M |
| Inter-assay variability | 5% | 15% |
| Intra-assay variability | <5% | 15% |
| Yield | 90% | 10% |
| Sample Volume | 0.1 ml | 0.5 - 1 ml |
| Processing Time (20 samples) | 3-4 hours | 1-2 days |

These results demonstrate the advantages of the present enzymatic assay as compared to the TLC/GC assay. The assay is linear from 0.2 µM to 1 µM of LysoPA concentration. In

addition, the enzymatic assays of the present invention provide high yield, increased sensitivity and rapid processing time.

## EXAMPLE II

## DETECTION AND OUANTITATION OF LYSOPC LEVELS IN HUMAN PLASMA AND SERUM

### Reagents

Lysophospholipase (LYPL) was purchased from Asahi Chemical Industry, Tokyo, Japan. Glycerophosphorylcholine phosphodiesterase (GPC-PDE), choline oxidase, and 4-aminoantipyrine (AAP) were purchased from Sigma Chemical Co., St. Louis, MO. Peroxidase was purchased from Boerhinger Mannheim, Indianapolis, IN. 3,5 Dichloro-2-hydroxybenzenesulfonic acid sodium salt (HDCBS) was purchased from Biosynth AG, Naperville, IL. All lipid standards and fatty acids were purchased from Avanti Polar Lipids, Alabaster, AL or Sigma Chemical Co.

### Sample Collection and Processing

Blood was collected and plasma was processed as described in Example I. For serum, blood was collected in silicone-coated Vacutainer tubes (Red Top) and was centrifuged under normal conditions. Serum and plasma was transferred to plastic tubes and stored frozen at -20$^0$C to -80$^0$C.

### Sample Preparation for the Enzymatic Assay

Approximately 35 µl plasma or serum was diluted 1:10 in sample buffer (1% Triton, 10 mM calcium chioride, 50 mM Tris pH 8.0) to a total volume of 350 µl.

Enzymatic Assay

In the well of a 96 well microtiter plate, 100 μl of the diluted lipid is aliquoted in replicate. To each well, 50 μl of LYPL (0.125 Units) /GPC-PDE (0.0125 Units) is added and incubated at $37^0$C for 10 minutes. This reaction produces glycerophosphorylcholine as an intermediate through LYPL digestion of LysoPC. The GPD-PDE then liberates G-3-P and choline from glycerophosphorylcholine. The plate is then blanked A505 in the ELISA reader. Next, 50 μl choline detection mix (0.15 Units choline oxidase, 0.5 Units peroxidase, 0.03% AAP, 0.125% HDCBS, 100 mM Tris pH 8.0) is added and incubated at $37^0$C for 15 minutes. The plate is then read at $A_{505}$.

Table II illustrates the results of the assay for LysoPC. The assay is linear from 5 to 200 μM LysoPC, sensitive to 5 μM LysoPC and exhibits low intra-assay and inter-assay variability.

TABLE II

ENZYME ASSAY TO DETET LYSOPC IN PLASMA

| | |
|---|---|
| Sensitivity | 5 μM |
| Linear Range | 5-200 μM |
| Intra-assay variability | 3.0% |
| Inter-assay variability | 6.0% |

These results show that LysoPC is easily detected in plasma or serum using the two-step enzyme assay of the invention. Similar results were obtained from plasma or serum from the same patient, demonstrating that the method is applicable to either plasma or serum. Typical

LysoPC levels in plasma or serum ranged from 50 μM to 500 μM. As a result, LysoPC can be determined in a 1:10 diluted sample using this assay.

## EXAMPLE III

## DETECTION AND OUANTITATION OF LYSOPA IN SAMPLES FROM PATIENTS HAVING CANCER

LysoPA levels were determined in plasma of both non-cancer subjects and patients having ovarian cancer. Blood was collected from female patients and was processed as described above in Example I. Plasma from the samples was prepared for the enzymatic assay of the invention as described above in Example I. The enzyme assay was performed as described above in Example I.

Average LysoPA levels for non-cancer and cancer patients as determined using the enzyme assay shows that average levels of LysoPA were significantly increased in the plasma of patients having ovarian cancer as determined using the methods of the invention.

In addition, levels of LysoPC and PC were determined from the plasma of patients with and without ovarian cancer using the enzyme assay as described above in Examples II and III. These results were combined and multipled to yield a multi-lipid diagnostic measurement. Levels of LysoPC and PC determined independently were 10 to 100% higher in ovarian cancer versus normal patients. Combining and multiplying LysoPA X LysoPC X PC levels for each sample yielded a measurement from 400% to 500% higher in ovarian cancer versus normal patients. These results suggest that the combinatorial approach may provide a more accurate assay for detecting conditions such as cancer associated with altered levels of lysophospholipids

and phospholipids by reducing the number of false positive and false negative results.

## EXAMPLE IV

## DETECTION AND OUANTIFICATION OF LYSOPA IN PATIENTS HAVING A BLEEDING DISORDER

LysoPA levels were determined as described above in Example I in 93 plasma samples from male and female patients over an age range of 1-80 years. Of the 93 samples, 17 of samples came from patients who were previously diagnosed with bleeding disorders *(i.e.* coagulopathy). LysoPA levels were determined. Patients having a bleeding disorder demonstrated significantly higher average LysoPA levels than those patients not having cancer or a bleeding disorder.

The results from the examples herein demonstrate that the methods of the invention can be used to detect altered lysophospholipid and phospholipids such as PC levels in patients having various disease conditions associated with such altered levels. Moreover, these results provide a new method for diagnosing disease conditions associated with altered levels of lysophospholipids in which levels of different phospholipids such as LysoPA and LysoPC in plasma or serum are multiplied to detect the disease condition.

Various publications are cited herein which are hereby incorporated by reference in their entirety.

As will be apparent to those skilled in the art in which the invention is addressed, the present invention may be embodied in forms other than those specifically disclosed above without departing from the spirit or potential characteristics of the invention. Particular

embodiments of the present invention described above are therefore to be considered in all respects as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims and equivalents thereof rather than being limited to the examples contained in the foregoing description.